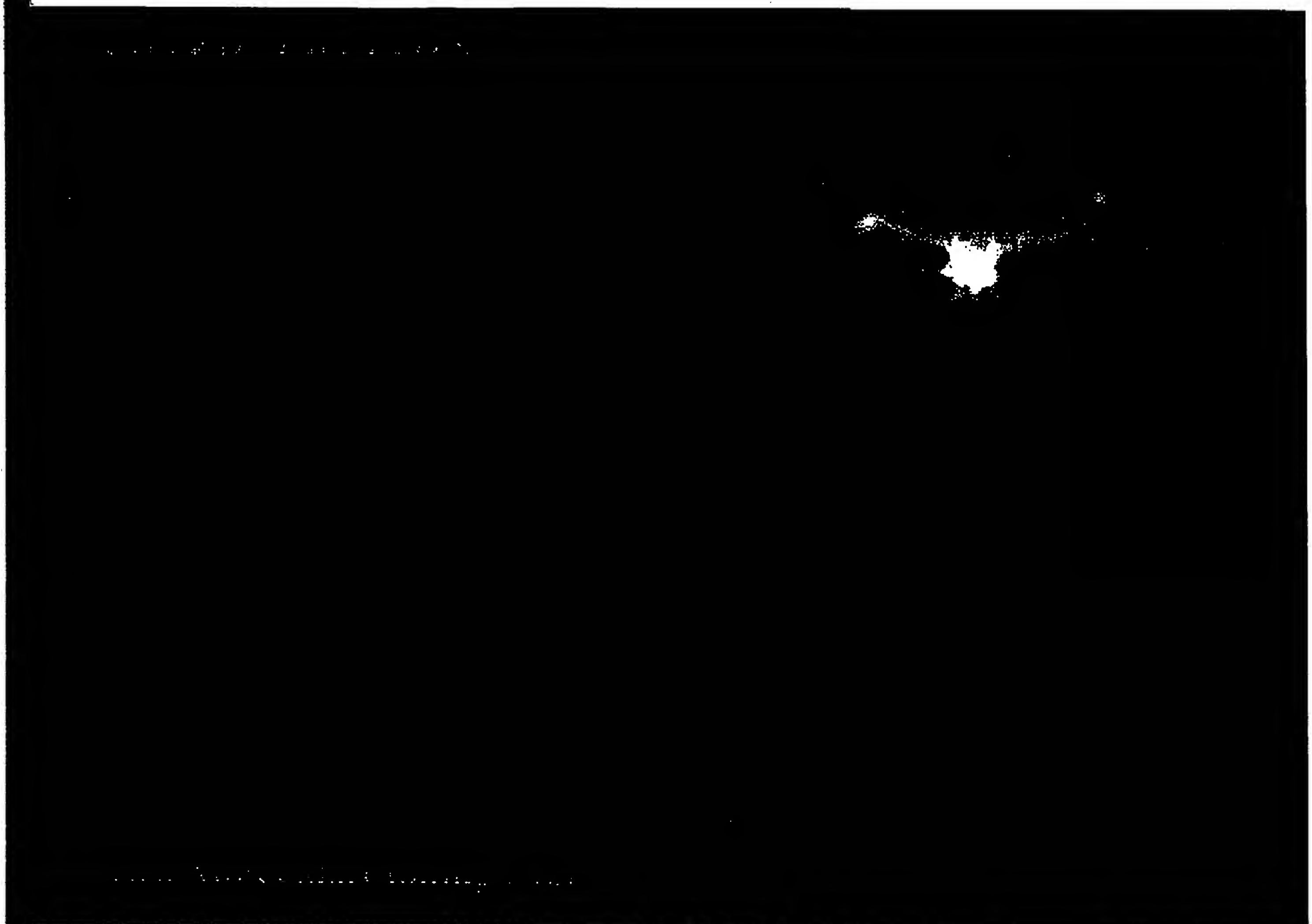
VOLUME 3

EXHIBIT 1

Molecular Cloning

A LABORATORY MANUAL



Sambrook and Russell

CSHL PRESS

Molecular Cloning

A LABORATORY MANUAL

THIRD EDITION

Printed in the United States of America

Front cover (paperback): The gene encoding green fluorescent protein was cloned from *Aequorea victoria*, a jellyfish found in abundance in Puget Sound, Washington State. This picture of a 50-mm medusa was taken on color film by flash photography and shows light reflected from various morphological features of the animal. The small bright roundish blobs in the photograph are symbiotic amphipods living on or in the medusa. The bright ragged area in the center is the jellyfish's mouth.

Bioluminescence from *Aequorea* is emitted only from the margins of the medusae and cannot be seen in this image. Bioluminescence of *Aequorea*, as in most species of jellyfish, does not look like a soft overall glow, but occurs only at the rim of the bell and, given the right viewing conditions, would appear as a string of nearly microscopic fusiform green lights. The primary luminescence produced by *Aequorea* is actually bluish in color and is emitted by the protein aequorin. In a living jellyfish, light is emitted via the coupled green fluorescent protein, which causes the luminescence to appear green to the observer.

The figure and legend were kindly provided by Claudia Mills of the University of Washington, Friday Harbor. For further information, please see Mills, C.E. 1999–2000. Bioluminescence of *Aequorea*, a hydromedusa. Electronic Internet document available at http://faculty.washington.edu/cemills/Aequorea.html. Published by the author, web page established June 1999, last updated 23 August 2000.

Back cover (paperback): A portion of a human cDNA array hybridized with a red fluor-tagged experimental sample and a green fluor-tagged reference sample. Please see Appendix 10 for details. (Image provided by Vivek Mittal and Michael Wigler, Cold Spring Harbor Laboratory.)

Library of Congress Cataloging-in-Publication Data

Sambrook, Joseph.
Molecular cloning : a laboratory manual / Joseph Sambrook, David W. Russell.-- 3rd ed.
p. ; cm
Includes bibliographical references and index.
ISBN 0-87969-576-5 (cloth) -- ISBN 0-87969-577-3 (pbk)
1. Molecular cloning--Laboratory manuals.
[DNLM: 1. Cloning, Molecular--Laboratory Manuals. QH 440.5 S187m 2001] I. Russell, David W. (David William), 1954- . II. Title.
QH442.2 .S26 2001
572.8--dc21

00-064380

10 9 8 7 6 5 4 3 2 1

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1x Tris-glycine electrophoresis buffer

25 mM Tris
250 mM glycine (electrophoresis grade) (pH 8.3)
0.1% (w/v) SDS

Prepare a 5x stock of electrophoresis buffer by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of deionized H_2O. Then add 50 ml of a 10% (w/v) stock solution of electrophoresis-grade SDS and adjust the volume to 1000 ml with H_2O.

Special Equipment

Power supply

A device capable of supplying up to 500 V and 200 mA is needed.

Vertical electrophoresis apparatus

The use of discontinuous buffer systems requires SDS-polyacrylamide electrophoresis to be carried out in vertical gels. Although the basic design of the electrophoresis tanks and plates has changed little since Studier (1973) introduced the system, many small improvements have been incorporated into the apparatuses. Standard vertical as well as minigel vertical electrophoresis systems for separation and blotting of proteins are now sold by many manufacturers (e.g., Life Technologies). Which of these systems to purchase is a matter of personal choice, but it is sensible for a laboratory to use only one type of apparatus. This type of standardization makes it easier to compare the results obtained by different investigators and allows parts of broken apparatuses to be scavenged and reused.

METHOD

Pouring SDS-polyacrylamide Gels

1. Assemble the glass plates according to the manufacturer's instructions.
2. Determine the volume of the gel mold (this information is usually provided by the manufacturer). In an Erlenmeyer flask or disposable plastic tube, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table A8-9. Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step.

   The concentration of ammonium persulfate that we recommend is higher than that used by some investigators. This eliminates the need to rid the acrylamide solution of dissolved oxygen (which retards polymerization) by degassing.
3. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Use a Pasteur pipette to carefully overlay the acrylamide solution with 0.1% SDS (for gels containing ~8% acrylamide) or isobutanol (for gels containing ~10% acrylamide). Place the gel in a vertical position at room temperature.

   The overlay prevents oxygen from diffusing into the gel and inhibiting polymerization. Isobutanol dissolves the plastic of some minigel apparatuses.
4. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionized H_2O to remove any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining H_2O with the edge of a paper towel.
5. Prepare the stacking gel as follows: In a disposable plastic tube, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in

Table A8-10. Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step.

The concentration of ammonium persulfate is higher than that used by some investigators. This eliminates the need to rid the acrylamide solution of dissolved oxygen (which retards polymerization) by degassing.

6. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Place the gel in a vertical position at room temperature.

   Teflon combs should be cleaned with H_2O and dried with ethanol just before use.

Preparation of Samples and Running the Gel

7. While the stacking gel is polymerizing, prepare the samples in the appropriate volume of 1x SDS gel-loading buffer and heat them to 100°C for 3 minutes to denature the proteins.

   Be sure to denature a sample containing marker proteins of known molecular weights. Mixtures of appropriately sized polypeptides are available from commercial sources

   Extremely hydrophobic proteins, such as those containing multiple transmembrane domains, may precipitate or multimerize when boiled for 3 minutes at 100°C To avoid these pitfalls, heat the samples for 1 hour at a lower temperature (45–55°C) to effect denaturation.

8. After polymerization is complete (30 minutes), remove the Teflon comb carefully. Use a squirt bottle to wash the wells immediately with deionized H_2O to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe.

   ▲ **IMPORTANT** Do not prerun the gel before loading the samples, since this procedure will destroy the discontinuity of the buffer systems.

9. Load up to 15 µl of each of the samples in a predetermined order into the bottom of the wells. This is best done with a Hamilton microliter syringe or a micropipettor equipped with gel-loading tips that is washed with buffer from the bottom reservoir after each sample is loaded. Load an equal volume of 1x SDS gel-loading buffer into any wells that are unused.

10. Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir). Apply a voltage of 8 V/cm to the gel. After the dye front has moved into the resolving gel, increase the voltage to 15 V/cm and run the gel until the bromophenol blue reaches the bottom of the resolving gel (~4 hours). Then turn off the power supply.

11. Remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Use an extra gel spacer to carefully pry the plates apart. Mark the orientation of the gel by cutting a corner from the bottom of the gel that is closest to the leftmost well (slot 1).

    ▲ **IMPORTANT** Do not cut the corner from gels that are to be used for immunoblotting.

12. At this stage, the gel can be fixed, stained with Coomassie Brilliant Blue or silver salts, fluorographed or autoradiographed, or used to establish an immunoblot, all as described on the following pages.